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Fecal Excretion of a Nonenveloped DNA Virus (TTV) Associated With Posttransfusion Non-A–G Hepatitis

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Five patients with type B or C hepatocellular carcinoma were found to be infected with a nonenveloped DNA virus (TTV) associated with posttransfusion hepatitis of non-A–G etiology. Paired feces and serum samples from these patients were tested for TTV DNA by polymerase chain reaction with seminested primers and their sequences were compared. TTV DNA was detected in sera from all of the patients, while it was detected in feces from three patients, including two with high viral titers in serum. When feces and serum from one patient were subjected to floatation ultracentrifugation in CsCl, TTV in feces banded at a peak density of 1.35 g/cm^3 and that in serum at 1.31–1.32 g/cm^3. TTV isolates in three pairs of feces and serum had the identical sequence of 222 base pairs. The excretion of TTV into feces indicates that TTV would be transmitted not only parenterally but also nonparenterally by a fecal–oral route. ***J. Med. Virol. 56:128–132, 1998.***

KEY WORDS: hepatitis viruses; genotypes; feces; Parvoviridae infections

INTRODUCTION

By means of representational difference analysis [Lisitsyn et al., 1993], we discovered a novel virus that is transmitted by transfusion in association with elevated transaminase levels [Nishizawa et al., 1997]. The virus was designated TT virus (TTV) after the patient from whom it was recovered. TTV is a nonenveloped, single-stranded DNA virus with a density of 1.31–1.32 g/cm^3 [Okamoto et al., 1998]. Despite being a DNA virus, TTV has a wide range of sequence divergence, allowing classification into genotypes (1 and 2) differing by ~30%, each of which divide into subtypes (a and b) differing by ~15% [Okamoto et al., 1998].

By use of PCR with universal primers deduced from sequence comparison of distinct genotypes and subtypes, the DNA of TTV is detected in 47% (9 of 19) and 46% (41 of 90) of patients with acute and chronic hepatitis of non-A–G etiology, respectively, much more frequently than in blood donors at 12% (34 of 290) [Okamoto et al., 1998]. TTV DNA is common in populations at increased risk for infection with blood-borne viruses, such as patients with hemophilia (19 of 28 or 68%) or on maintenance hemodialysis (26 of 57 or 46%), and abusers of intravenous drugs (14 of 35 or 40%). TTV may replicate in hepatocytes, because its DNA is detected in the liver in titers from 10 to 100 times higher than in the corresponding serum from some patients with chronic non-A–G hepatitis [Okamoto et al., 1998].

As a nonenveloped virus with a linear, single-stranded genomic DNA, TTV resembles the *Parvoviridae* among known animal viruses. Animal parvoviruses, such as feline parvovirus and mink enteritis virus, are shed into feces, and therefore infect by the fecal–oral route [Pattison, 1990]. Hence, TTV may be excreted into feces for a nonparenteral infection. In order to evaluate this possibility, DNA of TTV was determined in the feces from five patients who had the virus in the circulation.

MATERIALS AND METHODS

Patients

Paired feces and serum samples were obtained from five patients who were found with TTV DNA in serum. They all had hepatocellular carcinoma and included two patients with hepatitis B surface antigen (HBsAg), two with antibody to hepatitis C virus (anti-HCV), and

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one with both HBsAg and anti-HCV. HBsAg was determined by hemagglutination (MyCell, Institute of Immunology Co., Tokyo, Japan) and anti-HCV by a second-generation enzyme immunoassay (ELISA-II, Ortho Diagnostic Systems, Tokyo, Japan) using commercial kits.

Fecal specimens (5–10 g) were suspended at 15% (wt/vol) in Tris-HCl buffer (0.01 M, pH 7.5), centrifuged in a Hitachi Refrigerated Centrifuge (Hitachi Koki, Tokyo, Japan) at 1,600 × *g* for 30 min, and the supernatant was recovered. It was spun down in a high-speed micro refrigerated centrifuge (Tomy Seiko, Tokyo, Japan) at 6,200 × *g* for 5 min, and a clear supernatant was obtained.

Determination of TTV DNA

Nucleic acids were obtained from 50 or 200 μl of serum or fecal supernatant by treatment with proteinase K and sodium dodecyl sulfate (SDS) followed by extraction with phenol and chloroform after the method described previously [Okamoto et al., 1990]. Nucleic acids were dissolved in 20 μl of Tris-HCl buffer (10 mM, pH 8.0) supplemented with 1 mM EDTA (hereafter referred to as TE buffer), heated at 95°C for 15 min and cooled immediately on ice. TTV DNA was determined in a half portion of the extract by polymerase chain reaction (PCR) with seminested primers [Okamoto et al., 1998] and Perkin-Elmer AmpliTaq DNA Polymerase (Roche Molecular Systems, NJ) by the method described below. In essence, the first-round PCR was carried out for 35 cycles (94°C, 30 sec; 60°C, 45 sec; 72°C, 45 sec; with additional 7 min in the last cycle) with NG059 primer (sense: 5′-ACAGACAGAGGAGAAGGCAACATG-3′) and NG063 (antisense: 5′-CTGGCATTTTACCATTTCCAAAGTT-3′), and the second-round PCR for 25 cycles under the same conditions with NG061 (sense: 5′-GGCAACATGYTRTGGATAGACTGG-3′, where Y = T or C; R = A or G) and NG063. The product of the first-round PCR measured 286 base pairs (bp), and that of the second-round PCR 271 bp.

Nucleic acids extracted from feces and serum, as well as fractions of the floatation ultracentrifugation, were diluted serially 10-fold in TE buffer supplemented with 20 μg/ml glycogen (Boehringer Mannheim, Mannheim, Germany), and then determined for TTV DNA by PCR. The highest dilution (10^N) found positive was estimated, and it was converted to represent the titer per 1 ml of serum or fecal supernatant.

Ultracentrifugation in a Density Gradient of CsCl

Floatation ultracentrifugation was undertaken by the following procedures. Serum or fecal supernatant (0.8 ml) from patient 1 was mixed with 1.6 ml of 54% (wt/wt) CsCl in TE buffer supplemented with 150 mM NaCl, along with 10 μl of a serum containing DNA of hepatitis B virus (HBV) in high titer (10^8/ml), which served as a size marker. The mixture was delivered to an SW60 tube (Beckman, CA) with a capacity of 4.4 ml, and overlaid successively with 1 ml of 30% and 0.9 ml of 10% CsCl. The tube was centrifuged at 179,200 × *g* at 10°C for 39 hr, and then 200 μl fractions were aspirated from the surface. Each fraction was tested for the density by refractometer. Nucleic acids were extracted from 10 μl of each fraction, dissolved in 20 μl of TE buffer, and tested for TTV DNA by PCR. HBV DNA was sought, in a 1/100 portion of extracted nucleic acids, by PCR with primers deduced from the envelope gene, by the method described previously [Iizuka et al., 1992].

Equilibrium ultracentrifugation was carried out as follows. A density gradient was prepared in an SW60 tube with 0.8 ml each of 40%, 30%, 20%, and 10% CsCl. Serum or fecal supernatant (200 μl) from patient 1, along with 2 μl of the serum containing HBV DNA (10^8/ml), was layered onto the surface of gradient, and overlaid with 900 μl of TE buffer. The tube was centrifuged at 179,200 × *g* at 10°C for 44 hr, and then 200 μl fractions were recovered from the surface. Each fraction was tested for density, TTV DNA, and HBV DNA.

Determination of a Partial TTV DNA Sequence

Products of the second-round PCR were ligated to pT7BlueT-Vector (Novagen, WI), and using plasmid DNA extracted from transformed *E. coli,* both strands were sequenced with ThermoSequenase fluorescent-labeled primer cycle sequencing kit (Amersham International, Buckinghamshire, England). Sequence was determined on three clones each for respective PCR products, and the consensus sequence was adopted.

Computer Analysis of Nucleotide Sequences

Sequence analysis of TTV isolates was performed using ODEN program version 1.1.1 (National Institute of Genetics, Mishima, Japan) and Genetyx-Mac version 8.0 (Software Development, Tokyo, Japan). A phylogenetic tree was constructed by the unweighted pair-group method with arithmetic mean [Nei, 1987].

RESULTS

TTV DNA in Feces

TTV DNA was detected in feces from three of the five patients with hepatocellular carcinoma with HBsAg or anti-HCV, or both, who had TTV DNA in serum (Table I). The detection of fecal TTV DNA was dependent on the viral titers in serum. TTV DNA was detected in feces from two patients with viral titers at 10^4/ml and 10^2/ml in serum (patients 1 and 2), respectively, but only in one of the three patients with titer at 10^1/ml (patient 3). The highest TTV DNA titer (10^5/ml) was detected in the fecal extract from patient 1, who had the highest titer in serum (10^4/ml).

Buoyant Density of TTV in Feces

Serum and feces from patient 1, who had the highest TTV DNA titer in serum (10^4/ml), were subjected to floatation ultracentrifugation in a density gradient of CsCl (Fig. 1). TTV in feces banded at a density of 1.35 g/cm^3, a little higher than that in serum at 1.32 g/cm^3.

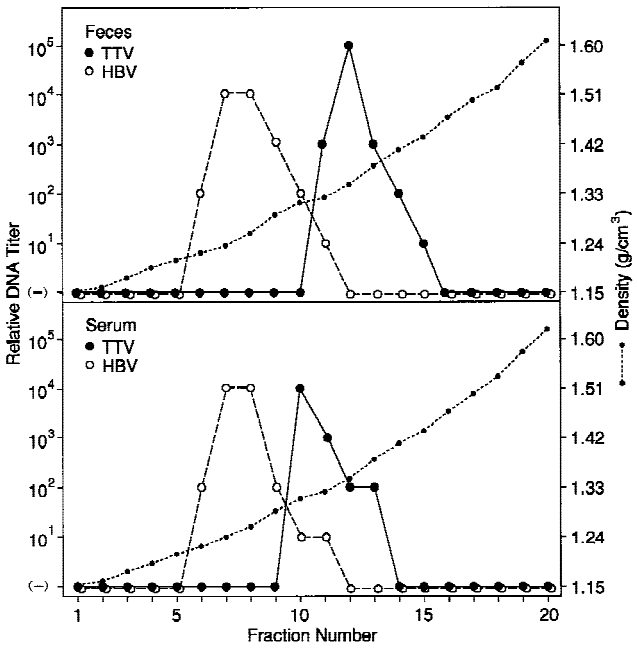

Fig. 1. Density-gradient fractionation of TTV in feces and serum from a patient. Fecal extract and serum containing TTV were subjected to fractionation in a density gradient with CsCl. HBV served as a size marker.

HBV serving as a size marker banded at 1.24–1.26 g/cm^3, as reported previously [Takahashi et al., 1976], thereby attesting to the validity of sizing by floatation ultracentrifugation.

The densities of TTV in feces and serum from patient 1 were confirmed by equilibrium ultracentrifugation, which gave the peak values of 1.35 g/cm^3 and 1.31–1.32 g/cm^3, respectively. HBV banded at 1.24–1.26 g/cm^3 in this ultracentrifugation.

Sequences of TTV DNA in Feces and Sera

A partial sequence of TTV DNA spanning 271 bp was determined for paired feces and serum samples from the three patients, and sequences of 222 bp (primer sequences at both ends excluded) were compared (Fig. 2). A complete identity was observed between TTV DNA sequences in paired feces and serum samples from each of the three patients.

TTV DNA sequences of the three patients were heterogeneous with each other, and differed from 15.3% to 38.7% in the nucleotide sequence and from 10.8% to 39.2% in the amino acid sequence. The sequence of patient 1 was similar in 97.7% and 98.0%, respectively, to the original N22 clone [Nishizawa et al., 1997] and the TA278 isolate for which a sequence of 3.7 kilobases was determined [Okamoto et al., 1998]; therefore, it was classified in genotype/subtype 1a [Okamoto et al., 1998]. The sequence of patient 2 was similar in 95.0% to that of the TX011 isolate of genotype/subtype 1b [Okamoto et al., 1998]; accordingly, it was deduced to be of 1b. The sequence of patient 3 was different from those of patients 1 and 2 and from TTV isolates of genotype 1. It resembled the sequences of TS003 isolate of 2a and NA004 isolate of 2b [Okamoto et al., 1998], but differed from them by 15.8% and 16.6%, respectively. Hence, a new subtype was assigned to it, which represented a third in genotype 2 and was provisionally designated 2c.

TABLE I. Titers and Genotypes/Subtypes of TTV in Paired Feces and Serum Specimens from Patients With Hepatocellular Carcinoma

	Fecal extract		Serum	
	Titer	Genotype/subtype[a]	Titer	Genotype/subtype[a]
Patients				
1	10^5/ml	1a	10^4/ml	1a
2	10^1/ml	1b	10^2/ml	1b
3	10^1/ml	2c	10^1/ml	2c
4	—[b]		10^1/ml	1a
5	—		10^1/ml	1a
Normal control	—		—	

[a]Genotypes and subtypes of TTV were determined by the method of Okamoto et al. [1998].
[b]Not detected.

DISCUSSION

There are patients with acute or chronic hepatitis in whom the etiology is unknown. Remarkably, most patients with fulminant hepatic failure nowadays are without markers of known hepatitis viruses [Wright et al., 1991; Fagan, 1994], and there are patients with chronic hepatitis with or without cirrhosis in whom the cause is not known [Kodali et al., 1994]. It is of particular note that most of these patients had not received transfusions or used illicit intravenous drugs. These lines of evidence point to viruses that are transmitted nonparenterally, most likely by a fecal–oral route, and that can induce hepatitis.

We found DNA of TTV, a possible hepatitis virus [Nishizawa et al., 1997; Okamoto et al., 1998], in feces from three of five patients who had TTV in the circulation. Fecal excretion of TTV seemed to be dependent on viral titers in serum, because TTV DNA was detected in two patients with serum titers of 10^4/ml and 10^2/ml, respectively, contrasting with the detection in only one of the three patients with a serum titer at 10^1/ml. TTV DNA in feces of the tested patient had a density in CsCl (1.35 g/cm^3) comparable with that in serum (1.31–1.32 g/cm^3). Hence, TTV in feces would be complete virions and infectious by fecal–oral transmission. This has to be verified by immune electron microscopy and experimental transmission to primates.

Fecal excretion of TTV was demonstrated in patients with hepatocellular carcinoma associated with HBV or HCV, or both, whose serum and fecal samples were readily available to us. It is necessary to test for TTV DNA in feces from individuals who carry TTV without any symptoms to extend the present findings.

TTV is a nonenveloped, single-stranded, and linear DNA virus with a high density, resembling parvoviruses. Due to its structural simplicity, it would be ex-

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                    *         *         *         *         *         *         *         80
N22 clone           CTAAGCAAAAAAAACATGAACTATGACAAACTACAAAGTAAATGCTTAATATCAGACCTACCTCTATGGGCAGCAGCATA
Patient 1 (feces)   ----------------------C------G----G-------------------------------------------
          (serum)   ----------------------C------G----G-------------------------------------------
Patient 2 (feces)   -----T------G-------A---------G-G--------G--TC--G--G-G--------A--G---------T----
          (serum)   -----T------G-------A---------G-G--------G--TC--G--G-G--------A--G---------T----
Patient 3 (feces)   ----CT---G-TG--TCTGC---CTCA---AC------C-----TC-C---GA-A-------C--G-----C----T---
          (serum)   ----CT---G-TG--TCTGC---CTCA---AC------C-----TC-C---GA-A-------C--G-----C----T---

                    *         *         *         *         *         *         *         160
                    TGGATATGTAGAATTTTGTGCAAAAAGTACAGGAGACCAAAACATACACATGAATGCCAGGCTACTAATAAGAAGTCCCT
                    ----------------------------------------G-----------------------------------------
                    ----------------------------------------G-----------------------------------------
                    ---T---T-------C--CT-T-----C---------AC----------------------A--G--------------T-
                    ---T---T-------C--CT-T-----C---------AC----------------------A--G--------------T-
                    C----TCAA-----AC--CAGC---GTA---------AC-------G-ACAC--CTG---ATGTG-T-----G--C----
                    C----TCAA-----AC--CAGC---GTA---------AC-------G-ACAC--CTG---ATGTG-T-----G--C----

                    *         *         *         *         *         *222
                    TTACAGACCCACAACTACTAGTACACACAGACCCCACAAAAGGCTTTGTTCCTTACTCTGTA
                    ------------------------------------------------------------T--
                    ------------------------------------------------------------T--
                    ---------C----------------------T--T--------C--A-----T---T--
                    ---------C----------------------T--T--------C--A-----T---T--
                    AC----TA--------GT---AC----ACA-----CTT-G---G-AC--G-----TAGCC-G
                    AC----TA--------GT---AC----ACA-----CTT-G---G-AC--G-----TAGCC-G
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Fig. 2. Sequence comparison of TTV DNA in paired feces and serum samples from the three patients. A sequence of 222 bp in the products of the second-round PCR (primer sequences at both ends excluded) is shown. The consensus sequences of three clones from each sample are shown. The sequence of the original N22 clone [Nishizawa et al., 1997] is indicated at the top. Dashes represent nucleotides identical to those in the N22 clone.

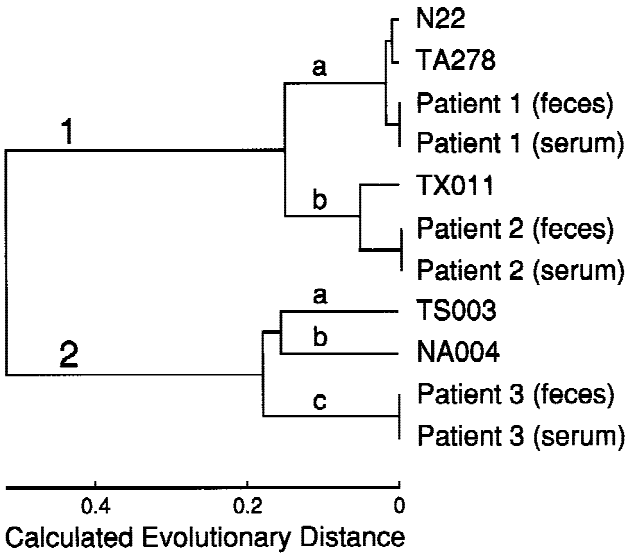

Fig. 3. A phylogenetic tree of TTV. The tree was constructed by comparison of a 222 bp sequence in TTV isolates, including the original N22 clone of genotype/subtype 1a, one each of clones of genotype/subtype 1a, 1b, 2a, or 2b representative of the 78 reported [Okamoto et al., 1998], as well as isolates from paired feces and serum samples from the three patients.

tremely resistant to inactivation. The parvovirus virion is stable between pH 3 and 9, and can stand heating at 56°C for 60 min [Berns, 1996]. Taken along with its excretion into the alimentary tract, a fecal–oral route of transmission would contribute to a wide spread of TTV among the community. This might explain why TTV DNA was detected with a high prevalence (12%) among symptom-free blood donors in Japan [Okamoto et al., 1998].

Unlike animal parvoviruses, human parvovirus B19 is not excreted into feces [Anderson et al., 1985]; TTV would be the first human parvovirus with a fecal excretion. The excretion of parvovirus-like virus into feces has been noted since 1973 [Paver et al., 1975], however, before the discovery of B19 in 1975 [Cossart et al., 1975]. It would be of interest to know how TTV is relevant to the parvovirus in human feces documented in the previous report.

Traditionally, viral hepatitis has been dichotomized into "serum hepatitis," which transmits parenterally and can eventuate in chronic disease, and "infectious hepatitis," which spreads nonparenterally by a fecal–oral route and rarely becomes chronic. TTV is transmitted parenterally, typically by transfusions, and can induce persistent infection [Nishizawa et al., 1997]. Hence, TTV DNA is frequent in populations at high risk for blood-borne viruses [Okamoto et al., 1998]. The shedding of TTV into feces from infected persons, as reported above, creates the possibility that it may also be transmitted nonparenterally. As such, TTV straddles over "serum hepatitis" and "infectious hepatitis," should its hepatitis-inducing capacity be established.

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